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Protein (TP) That is Involved in the Development of the Nervous System

The present invention relates to a protein (T protein) and to proteins related thereto which are involved in the development of the nervous system and are expressed in a tissue-specific and development-specific manner, to the below described variants of these proteins and to DNA sequences coding for these proteins. The present invention further relates to antibodies directed against these proteins or to fragments thereof as well as to antisense RNAs or ribozymes directed against the expression of these proteins. Finally, the present invention concerns medicaments and diagnostic methods in which the above-mentioned compounds are used.

Mutations in genes playing a part in the development and maintenance of the nervous system are of utmost scientific and economic significance, since diseases of the nervous system, in particular CNS, occur frequently, are often characterized by a severe, partly fatal disease process and can be treated only to a limited extent thus far. The increase in the life expectancy is accompanied by a drastic increase in neurological and psychic diseases. The latter greatly limit the quality of life of the affected persons and cause considerable costs for both the affected person and the public.

Isolating and analyzing genes specific to the nervous system offer a good possibility of studying diseases, such as schizophrenia, Alzheimer's disease, autism, manic depression

and mental retardation, and eventually of also being able to treat them.

The present invention is thus based on the technical problem of providing products by means of which disturbances in the development and function of the nervous system can be diagnosed and optionally be treated.

The solution to this technical problem is achieved by providing the embodiments characterized in the claims.

The subject matter of the present invention is thus a DNA sequence coding for a protein which is involved in the development and function of the nervous system, in particular the CNS, and is expressed in tissue-specific and development-specific manner, the DNA sequence comprising the following DNA sequences:

- (a) the DNA sequence of figure 1, figure 2, figure 3, figure 4, figure 5, figure 6, figure 7 or figure 8;
- (b) the DNA sequence of figure 9 or figure 10;
- (c) the DNA sequence of figure 11;
- (d) the DNA sequence of figure 12 or figure 13;
- (e) the DNA sequence of figure 14 or figure 15;
- (f) the DNA sequence of figure 16;
- (g) the DNA sequence of figure 17 or 18;
- (h) the DNA sequence of figure 19;
- (i) a DNA sequence hybridizing with (a), (b), (c), (d), (e), (f), (g) or (h);
- (j) variants, derivatives, precursors or fragments of the DNA sequence of (a), (b), (c), (d), (e), (f), (g), (h) or (i); or

(k) a DNA sequence differing from the DNA sequence of (a), (b), (c), (d), (e), (f), (g), (h), (i) or (j) due to the degeneration of the genetic code.

The present invention is based on the isolation of a human DNA sequence (referred to as gene "T" or T gene; see figures 1 to 8, which codes for protein TP), it turning out that the protein encoded by this DNA sequence is required in the nervous system. In this connection, the expression of the gene encoding this protein is increased in the nervous system. A sequence analysis showed that it is a new gene. Moreover, further genes could be isolated which have homologies to this gene (murine gene "T", figures 9 and 10; human gene "T2", figure 16; human gene "T3", figures 17 and 18; murine gene T2, figures 12 and 13; murine gene T3, figure 19). The T gene, T2 gene and T3 gene are members of the T (gene) family, as shown below, and originate preferably from vertebrates, such as man, mouse or rat. Defects in these genes limit the functions of the nervous system, in particular the CNS. These genes also perform an important function in the control of cell growth, and changes in these genes or their expression result in defects regarding the control of cell growth, e.g. also in tumor formation, in particular of the neuroblastoma. Small children up to the age of 8 are affected almost exclusively by this cancerous disease. The first symptoms already occur within the first 12 months of life in 25 to 30 percent of the cases. In the case of the neuroblastoma very young cells of the autonomous nervous system degenerate. Since these nerves extend along the rear side of the abdominal region and the chest, neuroblastomas usually occur in the regions of the stomach, pelvis, chest and neck. More than half the diseases start from the suprarenal marrow which is also formed by nerve cells. Symptoms which may refer in small

children to a neuroblastoma are nodes, swellings, bone pain, limping, tiredness, fever, paleness, sweating, obstinate or persistent cough, hematomas around the eye. A neuroblastoma can be diagnosed by a physician by means of blood tests, urine analyses and ultrasonic examinations and by the removal of biopsies from the tumor and an examination of bone marrow. As soon as the accurate location of the tumor is diagnosed, it is removed by means of an operation. However, the early formation of metastases creates a problem. By isolating and analyzing the T gene it is now possible to develop novel measures of diagnosing and treating the neuroblastoma. Due to this, it is possible to diagnose the cancerous disease early and establish forms of therapy promising better chances of recovery.

Mutations in genes of the T gene family also lead to a disturbed development and differentiation of the nervous system, in particular the brain. In many cases, this results in mental diseases, e.g. mental retardations or Alzheimer's disease. The T gene also plays an important role in the interconnection of individual regions of the brain, e.g. forebrain and midbrain. Mutations in this gene lead in some cases to schizophrenic diseases and syndromes of autism. By means of the human and murine genes it is possible to draw important fundamental conclusions as to the development of the nervous system and in particular the brain. Good approaches offer themselves as regards the research of pathologic changes of the nervous system and in particular the brain.

Patients can be examined more simply for possible mutations by means of the genomic sequences. The genomic sequences of the T gene are of advantage in particular when little (tumor) material is available for the analysis. By this it

is possible, for example, to examine even minute tumors for mutations in this gene. This also provides the possibility of checking a therapy (in particular radiation therapy and/or chemotherapy) for its being successful, since it is possible to detect tumor cells circulating in the blood by genomic primers which are specific to the genomic DNA using a PCR reaction.

The term "hybridizing" used in the present invention relates to conventional hybridization conditions, preferably to hybridization conditions which use 5xSSPE, 1 % SDS, 1xDenhardt's solution as the solution and where hybridization temperatures are between 35°C and 70°C, preferably 65°C. Following hybridization, washing is preferably carried out using first 2xSSC, 1 % SDS and then 0.2xSSC at temperatures between 35°C and 70°C, preferably of 65°C (regarding a definition for SSPE, SSC and Denhardt's solution see Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2<sup>nd</sup> edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989)). Stringent hybridization conditions are particularly preferred, as described in Sambrook *et al.*, *supra*, for example.

The terms "variants" or "fragment" used in the present invention comprise DNA sequences which differ from the sequences indicated in the figures by deletion(s), insertion(s), substitution(s) and/or other modifications known in the art or comprise a fragment of the original nucleic acid molecule, the protein or peptide encoded by these DNA sequences still having the above-mentioned properties. Therefore, functional equivalents, derivatives, precursors (bioprecursors) are counted among them. Derivatives are understood to mean e.g. mutation derivatives

(produced by deletions or insertions, for example), fusions, allele variants, muteins and splicing variants. Two select examples of such splicing variants are shown in figures 14 and 15. Methods of producing the above changes in the nucleic acid sequence are known to a person skilled in the art and are described in standard works of molecular biology, e.g. in Sambrook *et al.*, *supra*. The person skilled in the art is also capable of determining whether a protein encoded by a nucleic acid sequence modified in such a way still has the above-mentioned properties.

In a preferred embodiment, the present invention relates to a DNA sequence which encodes a protein comprising the amino acid sequence of figure 1, figure 9, figure 11, figure 12, figure 13, figure 14, figure 15, figure 16, figure 17, figure 18 or figure 19, the protein having the above-defined biological activity.

By lowering or inhibiting the expression of the above described DNA sequences it is possible to reduce or eliminate the synthesis of the proteins encoded by them, e.g. the T protein, which is desirable for certain states of a disease, for example. Therefore, another preferred embodiment of the present invention relates to antisense RNA, which is characterized in that it is complementary to the above DNA sequences and can reduce or inhibit the synthesis of the protein encoded by these DNA sequences and to a ribozyme, which is characterized in that it can bind specifically to part of the above DNA sequences and to the RNA transcribed by these DNA sequences and can cleave them so as to reduce or inhibit the synthesis of the protein encoded by these DNA sequences. These antisense RNAs and ribozymes are preferably complementary to a coding region of the mRNA. Based on the disclosed DNA sequences, the person

skilled in the art can produce and use suitable antisense RNAs. Suitable methods are described in EP-B1 0 223 399 or EP-A1 0 458, for example. Ribozymes are RNA enzymes and consist of a single RNA strand. They can cleave intermolecularly other RNAs, e.g. the mRNAs transcribed by the DNA sequences according to the invention. These ribozymes must, in principle, have two domains: (1) a catalytic domain and (2) a domain which is complementary to the target RNA and can bind thereto, which is a precondition for a cleavage of the target RNA. Based on the methods described in the literature, it is meanwhile possible to construct specific ribozymes which excise a desired RNA at a certain pre-select site (see e.g. Tanner et al., in: *Antisense Research and Applications*, CRC Press, Inc. (1993), 415-426).

The DNA sequences according to the invention or the DNAs encoding the above described antisense RNAs or ribozymes may also be inserted in a vector or expression vector. Thus, the present invention also comprises vectors or expression vectors containing these DNA sequences. The term "vector" relates to a plasmid (e.g. pUC18, pBR322, pBlueScript), to a virus or another suitable vehicle. In a preferred embodiment, the DNA molecule according to the invention is functionally linked in the vector to regulatory elements allowing the expression thereof in prokaryotic or eukaryotic host cells. Along with the regulatory elements, e.g. a promoter, such vectors contain typically a replication origin and specific genes which allow the phenotypic selection of a transformed host cell. The lac, trp promoter or the T7 promoter are counted among the regulatory elements for the expression in prokaryotes, e.g. *E. coli*, those for the expression in eukaryotes comprise the AOX1 or GALL promoter in yeast, and those for the expression in animal

cells include the CMV, SV40, RVS40 promoter, CMV or SV40 enhancer. Further examples of suitable promoters are the metallothionein I promoter and the polyhedrin promoter. In a preferred embodiment the vector contains the promoter of the human T gene or an ortholog of the T gene. Suitable expression vectors for *E. coli* are e.g. pGEMEX, pUC derivatives, pGEX-2T, pET3b and pQE-8, the latter being preferred. Suitable vectors for the expression in yeast comprise pY100 and Ycpad1, and suitable vectors for the expression in mammalian cells include pMSXND, pKCR, pEFBOS, cDM8 and pCEV4. Vectors derived from baculovirus for expression in insect cells, e.g. pAcSGHisNT-A, are also counted among the expression vectors according to the invention.

General methods known in the art can be used for constructing expression vectors which contain the DNA sequences according to the invention and suitable control sequences. These methods comprise e.g. *in vitro* recombination techniques, synthetic methods, and *in vivo* recombination techniques, as described in Sambrook *et al.*, *supra*, for example. The DNA sequences according to the invention can also be inserted in combination with a DNA coding for another protein or peptide, so that the DNA sequences according to the invention can be expressed in the form of a fusion protein, for example. These other DNAs are preferably reporter sequences which code for a reporter molecule comprising a detectable protein, e.g. a stain or coloring matter, an antibiotic resistance,  $\beta$ -galactosidase or a substances detectable by spectrophotometric, spectrofluorometric, luminescent or radioactive assays.

The present invention also relates to host cells containing the above described vectors. These host cells comprise

bacteria (e.g. the *E. coli* strains HB101, DH1, x1776, JM101, JM109, BL21 and SG13009), fungi, e.g. yeasts, preferably *S. cerevisiae*, plant cells, insect cells, preferably sf9 cells, and animal cells, preferably cells from vertebrates or mammals. Preferred mammalian cells are CHO, VERO, BHK, HeLa, COS, MDCK, 293 or WI38 cells. Methods of transforming these host cells for the phenotypic selection of transformants and for the expression of the DNA molecules according to the invention using the above-described vectors are known in the art.

The genes belonging to the sequences according to the invention can be amplified by suitable primer sequences. The primer sequences indicated in figure 20 are particularly suited for amplification of genes T2 and T3.

The present invention also relates to the proteins encoded by the DNA sequences according to the invention and to methods of producing the protein encoded by the DNA sequences according to the invention. The person skilled in the art is familiar with conditions of culturing transformed or transfected host cells. The method according to the invention comprises the culturing of the above described host cells under conditions which allow the expression of the protein (or fusion protein) (preferably stable expression) and the collection of the protein from the culture or from the host cells. Suitable purification methods (e.g. preparative chromatography, affinity chromatography, e.g. immunoaffinitychromatography, HPLC, etc.) are generally known.

The proteins according to the invention preferably comprise the amino acid sequences shown in figure 1, figure 9, figure 11, figure 12, figure 13, figure 14, figure 15, figure 16,

figure 17, figure 18 or figure 19 or represent fusions, fragments, derivatives or precursors (bioprecursors) thereof, the above mentioned properties being maintained within the meaning of functional equivalents. As to the definitions of these terms, reference is made to the respective explanations above. Derivatives are understood to mean in particular the changed proteins or peptides which differ from the sequences shown in the figures by conservative amino acid substitutions or contain non-conserved amino acid substitutions that do not change the function of the T proteins to a substantial degree.

The following amino acid motives have been identified by Inventors. They are suited to identify formerly unknown proteins which belong to the T/T2/T3 family according to the invention and a protein superfamily from pore membrane proteins and filament-binding proteins.

Motive 1:

(A, T) (I, P, V) (L, T) (G, A, Q) (L, V) XXX (L, V)

Motive 2:

IYTDWAN

Motive 3:

AXXXXXXXGXXXXXXAXXXXXXXXXXXXXXXQ

Motive 4:

SXXXXDX(12,20)KX(17,22)AXXXXXXXL

Motive 5:

IYTDWANXXLX(K, R)

Motive 6:

KX(18,21)AXXXXXXXLX(15,24)S

Motive 7:

NX(3,11)SXXXAXXXXXXXL

Explanation: X stands for every amino acid

(A, T) means amino acid A or T at this site

X(2,4) denotes two to four Xs at this site

Another preferred embodiment of the present invention relates to antibodies against the above described proteins according to the invention or to a fragment thereof. These antibodies may be monoclonal, polyclonal or synthetic antibodies or fragments thereof. In this connection, the term "fragment" means all parts of the monoclonal antibody (e.g. Fab, Fv or "single chain Fv" fragments) which have an epitope specificity the same as that of the complete antibody. The person skilled in the art is familiar with the production of such fragments.

The antibodies according to the invention are preferably monoclonal antibodies. The antibodies according to the invention can be produced according to standard methods, the protein encoded by the DNA sequences according to the invention or a synthetic fragment thereof serving as an immunogene. Methods of obtaining monoclonal antibodies are known to the person skilled in the art and comprise e.g. as a first step the production of polyclonal antibodies using the proteins according to the invention or fragments thereof (synthetic peptides, for example) as an immunogene for immunizing suitable animals, e.g. rabbits or chickens, and the collection of the polyclonal antibodies from the serum or egg yolk.

For example, cell hybrids from cells producing antibodies and tumor cells from bone marrow are then produced and cloned. Thereafter, a clone is selected which produces an antibody specific to the antigen used. This antibody is then produced. Examples of cells producing antibodies are spleen cells, lymph node cells, B lymphocytes, etc. Examples of animals which can be immunized for this purpose are mice,

rats, horses, goats and rabbits. The myeloma cells can be obtained from mice, rats, humans or other sources. The cell fusion can be carried out by the generally known method developed by Köhler and Milstein, for example. The hybridomas obtained by cell fusion are screened using the antigen according to the enzyme-antibody method or according to a similar method. Clones are obtained with the boundary dilution method, for example. The resulting clones are implanted intraperitoneally into BALB/c mice, for example, the mouse ascites is removed after 10 to 14 days, and the monoclonal antibody is purified by known methods (e.g. ammonium sulfate fractionation, PEG fractionation, ion exchange chromatography, gel chromatography or affinity chromatography).

In a particularly preferred embodiment, said monoclonal antibody is an antibody originating from an animal (e.g. mouse), a humanized antibody or a chimeric antibody or a fragment thereof. Chimeric antibodies similar to human antibodies or humanized antibodies have a reduced potential antigenicity, however, their affinity is not lowered over the target. The production of chimeric and humanized antibodies or of antibodies similar to human antibodies has been described in detail (see e.g. Queen *et al.*, Proc. Natl. Acad. Sci., U.S.A. 86 (1989), 10029, and Verhoeyan *et al.*, Science, 239 (1988), 1534). Humanized immunoglobulins have variable framework regions which originate substantially from a human immunoglobulin (designated acceptor immunoglobulin) and the complementarity of the determining regions which originate substantially from a non-human immunoglobulin (e.g. from a mouse) (designated donor immunoglobulin). The constant region(s) originate(s), if available, also substantially from a human immunoglobulin. When administered to human patients, humanized (and the

human) antibodies have a number of advantages over antibodies from mice or other species: (a) the human immune system should not regard the framework or the constant region of the humanized antibody as foreign and therefore the antibody response to such an injected antibody should be less than to that to a completely foreign mouse antibody or a partially foreign chimeric antibody; (b) since the effector region of the humanized antibody is human, it might interact better with other parts of the human immune system, and (c) injected humanized antibodies have a half life which is substantially equivalent to that of human antibodies occurring in nature, which permits the administration of doses smaller and less frequent as compared to antibodies of other species.

The antibodies according to the invention can be used for the immunoprecipitation of the above discussed proteins, for the isolation of related proteins from cDNA expression libraries or for the below indicated purposes (diagnosis/therapy), for example.

The present invention also relates to a hybridoma which produces the above described monoclonal antibody.

In a preferred embodiment, the present invention relates to antibodies against the peptides of genes T2 and T3 listed separately (cf. figure 20).

It has been found that the below peptide can be used specifically for generating antibodies against the T protein. The amino acid sequence of the suitable peptide reads as follows:

EKGEDPETRRMRTVKNIAD

The present invention makes possible to study disturbances in the development and function of the nervous system on a genetic level. These disturbances comprise *inter alia* neurological and psychiatric diseases (*inter alia* Alzheimer's disease, Parkinson's disease, schizophrenia, manic-depressive diseases, autism, mental retardations), injuries of the nervous system, innate damage of the nervous system or degenerative diseases of the nervous system. The invention also enables the treatment of cancer, *inter alia* of tumors of the nervous system, such as neuroblastoma, astrocytoma, glioblastoma, medulloblastoma. This diagnosis cannot only be made postnatally but already prenatally. It can be detected by means of the DNA sequence according to the invention or probes or primers derived therefrom whether mammals, in particular humans, contain a gene which codes for and/or expresses the protein according to the invention or whether this gene results in a mutated form of the protein which is no longer biologically active. For this purpose, the person skilled in the art can carry out common methods, such as reverse transcription, PCR, LCR, hybridization and sequencing. The antibodies according to the invention are also suited e.g. for diagnosis, i.e. for detecting in a sample the presence and/or concentration of the protein according to the invention, a shortened or extended form of the protein, etc. The antibodies can be bound e.g. in immunoassays in liquid phase or to a solid carrier. In this case, the antibodies can be labeled in various ways. Suitable markers and labeling methods are known in the art. Examples of immunoassays are ELISA and RIA.

Thus, the present invention also relates to a diagnostic method for detecting a disturbed expression of the protein

according to the invention or for detecting a changed form of this protein, in which a sample is contacted with the DNA sequences according to the invention or the antibody according to the invention or the fragment thereof and then it is determined directly or indirectly whether the concentration of the protein and/or its amino acid sequence differs from a protein obtained from a healthy patient.

The present invention also allows to carry out therapeutic measures in connection with the above discussed disturbances, i.e. the above described inventive DNA sequences, antisense RNAs, ribozymes and antibodies can also be used for producing a medicament, e.g. for controlling the expression of the protein according to the invention, or for exchanging a mutated form of the gene by a functional form and thus also for the production of a medicament for preventing or treating diseases of the nervous system, in particular tumoral diseases of the CNS. For example, the protein according to the invention can be introduced into mammals, in particular humans, by common measures. For this purpose, it may be favorable to link the protein to a protein which is not considered foreign by the respective body, e.g. transferrin or bovine serum albumin (BSA). An inventive DNA sequence, antisense RNA or ribozyme can also be introduced into mammals, in particular humans, and expressed. By means of an antibody according to the invention it is possible to control and regulate the expression of the protein (TP) according to the invention or the related proteins.

Thus, the present invention also relates to a medicament which contains the above described DNA sequences, antisense RNA, the ribozyme, the expression vector, the protein according to the invention or the antibody or the fragment

thereof. This medicament contains, optionally in addition, a pharmaceutically compatible carrier. Suitable carriers and the formulation of such medicaments are known to the person skilled in the art. Suitable carriers are e.g. phosphate-buffered common salt solutions, water, emulsions, e.g. oil-in-water emulsions, wetting agents, sterile solutions, etc. The medicaments can be administered orally or parenterally. The topical, intra-arterial, intra-muscular, subcutaneous, intramedullary, intrathekal, intraventricular, intravenous, intraperitoneal or intranasal administration are counted among the methods for the parenteral administration. The suitable dose is determined by the attending physician and depends on various factors, e.g. on the age, sex and weight of the patient, the stage of the disease, the kind of administration, etc.

The above described nucleic acids are preferably inserted in a vector suitable for gene therapy and introduced into the cells under the control of a tissue-specific vector, for example. In a preferred embodiment, the vector containing the above described nucleic acids is a virus, e.g. an adenovirus, vaccinia virus or adenovirus. Retroviruses are particularly preferred. Examples of suitable retroviruses are MoMuLV, HaMuSV, MuMTV, RSV or GaLV. For the purposes of gene therapy, the nucleic acids according to the invention can also be transported to the target cells in the form of colloidal dispersions. They comprise liposomes or lipoplexes, for example (Mannino *et al.*, *Biotechniques* 6 (1988), 682).

Finally the present invention relates to a diagnostic kit for carrying out the above described diagnostic method, which contains a DNA sequence according to the invention or the above described antibody according to the invention or a

fragment thereof. Depending on the kind of the kit, the DNA sequence or the antibody or the fragment thereof can be immobilized.

Sequences of the T genes can be applied to nylon membranes or glass carriers and hybridized with complex cDNA samples from tumors and pertinent normal tissues or diseased and pertinent healthy tissue. This enables the (fully automated) detection of the expression of these genes. The sequences used for this purpose can be e.g. the entire cDNA sequence or short sequence segments, e.g. 10-15 bp oligomers (see *inter alia* figure 20). Having determined the expression of the T genes, the therapy, *inter alia* the cancer therapy, can be selected deliberately according to the respective individual situation of the patient or can be adapted thereto. Genes whose changed expression influence already now the treatment of the patient are the N-myc gene in the case of neuroblastoma, for example. By detecting the expression of the T genes it is thus possible to adapt the treatment very quickly and efficiently to the respective requirements and in this way it contributes essentially to the improved therapy.

The isolation and characterization of the human gene according to the invention and in particular of the mouse homologues thereof also allow to establish an animal model, which is very valuable for the further study of diseases of the nervous system and of cancerous diseases on a molecular level. The subject matter of the present invention thus also relates to a non-human mammal whose T gene or T2 or T3 gene is changed, e.g. by inserting a heterologous sequence, in particular a selection marker sequence.

The expression "non-human mammal" comprises any mammal whose T gene or T2 or T3 gene can be changed. Examples of such mammals are mouse, rat, rabbit, horse, cattle, sheep, goat, monkey or ape, pig, dog and cat, with mouse being preferred.

The expression "T gene or T2 or T3 gene which is changed" signifies that a change of the gene structure or the gene sequence is carried out by standard methods in the corresponding gene occurring naturally in the non-human mammal. This can be achieved *inter alia* by introducing a deletion of about 1-2 kb, at the place of which a heterologous sequence, e.g. a construct for mediating antibiotic resistance (e.g. a "neo cassette") is introduced. Heterologous sequences allowing to carry out time-specific and tissue-specific deletions *in vivo* can also be inserted in the T gene. Furthermore, heterologous sequences allowing to track the expression of the T gene *in vivo* can be introduced into the T gene. This can be done *inter alia* by inserting a sequence coding for the GFP (green fluorescent protein) protein inside an exon or as an independent exon. These methods are generally described by Schwartzberg *et al.*, Proc. Natl. Acad. Sci., U.S.A., Vol. 87, pages 3210-3214, 1990, to which reference is made herein.

In particular, the modification can be described and carried out as follows. Figure 9 represents part of the cDNA sequence of the T gene of a mouse. Illustration 10 shows an intron sequence of the T gene of a mouse, which is flanked by two exons. These murine sequences can then be used for the deliberate change of the murine T gene. For example, the splicing sequences of the intron can be deleted or changed such that the T gene is no longer spliced correctly. By incorporating a splicing acceptor sequence of another exon of the murine T gene into the intron sequence it is possible

to insert in this intron a sequence which is recognized as exon and is spliced to the T gene exon upstream thereof. This inserted sequence may be an exon, for example, which encodes the EGFP protein (EnhancedGreenFluorescentProtein). As a result, the original murine T gene becomes a fusion protein comprising the EGFP protein. Thus, a mouse can preferably be generated, which allows to track the expression of the T gene *in vivo*. The inserted sequence can be designed at its end (e.g. PolyA signal, splicing signals, etc.) such that no further exons of the T gene are spliced to the inserted exon or the spliced exon can no longer be translated. As a result, a deletion of the murine T protein forms on the C-terminal end or a premature discontinuance of the reading frame, and an (at least partial) inactivation of the protein function of the murine T gene can be achieved. It is also possible to insert, as new exon sequences, sequences which yield an mRNA sequence where this new mRNA sequence is localized at the 3' end. By suitable sequences it is then possible to achieve a change in the stability of the mRNA or a changed localization in the cell. The accompanying phenotypes of the thus modified mice can then result in important conclusions drawn on the function of the T gene. These mice can then also be used for detecting new active substances compensating the functional loss of the T gene.

In another preferred embodiment, the sequence of figure 13 is used for the production of a knock-out mouse. Figure 13 describes a murine sequence of gene T2. The elimination of the murine T2 genes can in this connection be achieved in different ways. For example, the splicing sequence (GT, underlined in figure 13) can be deleted or changed such that the T2 gene is no longer spliced correctly. In addition, by incorporating a splicing acceptor sequence of another exon

of the murine T2 gene into the following intron sequence it is possible to insert in this intron a sequence which is detected as exon and spliced to the T2 gene exons upstream thereof. This inserted exon may be e.g. an exon which codes for the EGFP protein. Due to this, the original murine T2 gene becomes a fusion protein which carries the EGFP protein at the C terminus. In this way, a mouse can be generated which allows to track the expression of the T2 gene *in vivo*. The inserted sequence can be designed at its end (e.g. PolyA signal, etc.) such that no further exons are spliced to the inserted exon by the T2 gene. A deletion of the murine T2 protein forms at the C-terminal end and an (at least partial) inactivation of the protein function of the murine T2 gene can be achieved. Such sequences can also be inserted as new exon sequences which yield an mRNA sequence in which at the 3' end this new mRNA sequence is localized. By means of suitable sequences it is then possible to achieve a change in the stability of the mRNA or a changed localization in the cell. The accompanying phenotypes of the thus changed mice can then lead to important conclusions as to the function of the T2 gene. These mice can also be used for detecting new active substances which compensate the functional loss of the T gene.

Furthermore, a mammal can be generated comprising a change in the T3 gene. The sequence in figure 19 represents part of the murine cDNA sequence of the T3 gene. Deliberate changes in the T3 gene of a mouse can be achieved by deletions or insertions. The inserted sequence can be an exon, for example, which codes for the EGFP protein. As a result, the original murine T3 gene becomes a fusion protein which carries the EGFP protein at the C terminus. Thus, a mouse can be generated which allows to track the expression of the T3 gene *in vivo*. The inserted sequence can be designed at

its end (e.g. PolyA signal, etc.) such that no further exons are spliced from the T3 gene to the inserted exon. A deletion of the murine T3 protein thus forms on the C-terminal end and an (at least partial) inactivation of the protein function of the murine T3 gene can be achieved. It is also possible to insert, as new exon sequences, sequences which yield an mRNA sequence where this new mRNA sequence is localized at the 3' end. By suitable sequences it is then possible to achieve a change in the stability of the mRNA or a changed localization in the cell. The accompanying phenotypes of the mice changed in this way can then lead to important conclusions as to the function of the T3 gene. These mice can then also be used for discovering new active substances which compensate the functional loss of the T3 gene.

Another subject matter of the present invention are cells which are obtained from the above non-human mammal. These cells can be present in any form, e.g. in a primary or long-term culture.

A non-human mammal according to the invention can be provided by common methods. A method is favorable which comprises the steps of:

- (a) producing a DNA fragment, in particular a vector, containing a changed T, T2 or G3 gene, the gene having been modified by inserting a heterologous sequence, in particular a selectable marker;
- (b) preparing embryonal stem cells from a non-human mammal (preferably a mouse);

- (c) transforming the embryonal stem cells from step (b) with the DNA fragment from step (a), the T gene in the embryonal stem cells being changed by homologous recombination with the DNA fragment from (a);
- (d) culturing the cells from step (c);
- (e) selecting the cultured cells from step (d) for the presence of the heterologous sequence, in particular the selectable marker;
- (f) producing chimeric non-human mammals from the cells from step (e) by injecting these cells into mammalian blastocysts (preferably mouse blastocysts), transferring the blastocysts to pseudo-pregnant female mammals (preferably mouse) and analyzing the resulting offspring for a change of the T gene.

In step (c), the mechanism of homologous recombination (cf. R.M. Torres, R. Kühn, Laboratory Protocols for Conditional Gene Targeting, Oxford University Press, 1997) is used to transfect embryonal stem cells. The homologous recombination between the DNA sequences present in a chromosome and new added cloned DNA sequences enable the insertion of a cloned gene in the genome of a living cell in place of the original gene. Using embryonal germ cells, animals which are homozygous for the desired gene or the desired gene portion or the desired mutation can be obtained via chimeras by this method.

The expression "embryonal stem cells" comprises any embryonal stem cells of a non-human mammal, suited to mutate the T gene. The embryonal stem cells originate preferably from a mouse, in particular the cells E14/1 or 129/SV.

The expression "vector" comprises any vector which by recombination with the DNA of embryonal stem cells enables a change of the T, T2 or G3 gene. The vector preferably has a marker by means of which selection can be made for existing stem cells in which the desired recombination has been made. Such a marker is the loxP/tk neo cassette, for example, which can be removed by means of the Cre/loxP system from the genome again.

The person skilled in the art also knows conditions and materials serving for carrying out steps (a) - (f).

By means of the present invention a non-human mammal is provided whose T, T2 or T3 gene is changed. This change may be an elimination of the gene expression-regulating function. Using such a mammal or cells therefrom it is possible to study selectively the gene expression-controlling function of the TP protein. It is also possible by this to find substances, medicaments and therapy approaches by which selective influence on the controlled function is possible. The present invention therefore provides a basis for influencing the most differing diseases. Such diseases are e.g. limitations of the CNS functions which cover mental retardations or the induction of cancer resulting from deficiencies in the control of cell proliferation.

Inventors found out in the sequence analysis that the T2 gene in the coding region of the cDNA sequence contains CGG trinucleotides which are known to be sensitive to methylation. The T2 gene thus has in the coding region (N-terminal region of the protein which has no homology to the T protein or T3 protein) a methylation-sensitive and

unstable sequence which results in the failure of the gene accompanied by a mental retardation and uncontrolled cell growth, such as cancer.

All the three genes of the T family play a major role in the case of tumors. The T gene is affected in many tumors by genomic rearrangements. For example, in neuroblastomas genomic changes in the DNA of tumors can be found as compared to the DNA of the accompanying healthy tissue. The expression of the T gene, e.g. in tumors of the brain, is also changed. A strongly changed expression can be found *inter alia* in the advanced stages of glioblastomas. Tumor-specific changes of the expression of the T gene and the occurrence of the T protein can also be detected in meningiomas.

In many tumoral diseases, the T2 gene also undergoes genomic rearrangements, and a changed expression can be detected in tumors. For example, in melanomas and lung tumors genomic rearrangements of the T2 gene can be detected. Expression differences are also detectable in gliomas, glioblastomas, astrocytomas and PNETs (Primitive Neuro-Ectodermal Tumors), for example.

In many tumors, the T3 gene also undergoes genomic rearrangements and expression changes. Rearrangements can be detected in colon carcinomas, for example. Expression differences are detectable *inter alia* in gliomas, glioblastomas, astrocytomas and PNETs (Primitive Neuro-Ectodermal Tumors).

By isolating and accurately analyzing the T gene, Inventors now have found that the T protein has a certain relationship to proteins which perform completely different functions in

the cell. The sequence analysis of these proteins showed that the genes coding for these proteins are likely due to a common precursor gene or to similar precursor genes. Proteins such as the POM121 protein (Hallberg et al., J. Cell Biol. 122, pages 513-522, 1993) belong to this superfamily. It is one of two known nuclear pore membrane proteins in vertebrates. The CLIP-170 protein which binds vesicles and other organelles within the cell to microtubuli (Pierre et al., Cell 70, pages 887-900, 1992) also belongs to this family. The unexpected discovery that genes which perform such different tasks inside the cell belong to a common protein superfamily is extremely surprising and even inconsistent at first sight. However, when the functions of the individual genes are analyzed, two main functions of these proteins can be derived. The CLIP-170 protein binds to microtubuli, the newly isolated T proteins and the POM121 protein are localized in the nuclear core complex. Due to the properties of these proteins, Inventors propose that this protein superfamily be referred to as POMIC protein superfamily. POMIC shall, in this connection, stand for pores and/or microtubuli-binding protein. Based on the isolation and analysis of the T gene, two paralogs of the T gene could be isolated, namely the T2 and T3 genes which are described in more detail above. As regards evolution and function, the family of the T proteins is between the CLIP (cytoplasmic linker protein-170) and the POM121 protein. This intermediate position is also supported by the sequence analysis and the putative protein structure. The nuclear pore membrane protein POM121 has no marked coiled-coil structure whereas the CLIP-170 protein shows a very distinct coiled-coil structure between the N-terminus and C-terminus (cf. figure 29). Coiled-coil structures exist in the family of T proteins, however, they are clearly less marked than in CLIP-170. A similar intermediate position is adopted by the

family of T proteins with respect to the occurrence of hydrophobic domains. The POM121 protein has a hydrophobic domain at the N-terminus which is introduced into the nuclear membrane, and the protein is positioned in the nuclear pore. The CLIP-170 protein has no distinct hydrophobic domain. The T protein and the T3 protein, however, have a hydrophobic domain with three hydrophobic partial regions (cf. figure 30). The exchange of the N-terminus in the T2 protein as compared to the evolutionary basic form resulted in a loss of this distinct hydrophobic domain. Yet all three T proteins have in common the very similar structure of the C-terminus. The T3 protein is most similar to the T protein within the T protein family. However, the T3 protein also has undergone a change in the course of evolution. The N-terminus was changed as compared to the T protein by insertion of about 400 amino acids. This insertion resulted in another coiled-coil structure as compared to the otherwise very similar T protein. The T protein and the T3 protein perform functions in the nuclear membrane-localized form, which are similar to those of POM121. However, it is interesting that in the course of evolution there was a loss of part of the C-terminus in the POM121 protein. As compared to the POM121 protein, the T proteins have a longer C-terminus. Due to this longer C-terminus many interactions with other proteins are possible. In this connection, it is also worth mentioning that a leucine-zipper structure was discovered in the T protein, which facilitates interactions with other proteins. The family of T protein plays an important role in the mediation of interactions between cell organelles and filaments, *inter alia* microtubuli. Microtubuli play an important role e.g. in nerve cells; in the case of axons, for example, the plus ends of the microtubuli face away from the cell body whereas the microtubuli of dendrites have both orientations. This

cell polarity is of major importance for the functioning of a cell or living being. Microtubuli also provide an efficient organelle transport, and they are of essential significance for the general organization of membrane structures in a cell. The T proteins perform an important mediator function between membrane structures and microtubuli. The T gene and the T3 gene perform their function in particular as a membrane protein in the nuclear pore whereas the T2 protein acts particularly as a cytoplasmic protein.

Due to the findings of Inventors the T gene and the T3 gene are part of the nuclear pore complex. Nuclear pore complexes (NPCs) are extremely complicated structures which mediate the bi-directional transport of macromolecules between the nucleus and the cytoplasm. The nuclear pore complex is embedded in the nuclear envelope and encases a central channel with a structure only defined insufficiently thus far. Peripheral structures, short cytoplasmic filaments and a basket-like structure are attached on both sides of the central nuclear pore complex. This basket-like structure interacts with molecules which pass through the nuclear pore complex. The mechanism of synthesizing nuclear pore complexes is hardly understood thus far. In addition, it has been found when observing cells passing through mitosis that the nuclear envelope is dissolved deliberately and their components, including the nuclear pore proteins, are distributed over the mitotic cytoplasm. At the end of mitosis, all these components are used again to form the nuclear envelope of the daughter cells. Due to the detailed analysis of the gene T, Inventors found that the N-terminal half of the T protein is weakly homologous to the pore membrane protein POM121. The homology covers the entire region of the POM121 protein and has an identity of about 18

% on a protein level so that the DNAs underlying these proteins should not hybridize with one another, even under hardly stringent conditions. As regards the formation and structure of the nuclear pore, the T protein according to the invention plays a very fundamental role. In a detailed analysis of the protein, a lipophilic domain could be detected at the N-terminus of the T protein. However, this sequence has no homology to the lipophilic sequence of the POM121 protein. There is also a short segment of amino acids which might serve as a signal sequence before the lipophilic domain in the T protein. In order to find out whether this putative signal sequence and the lipophilic domain are involved *in vivo* in the incorporation into the nuclear membrane, various constructs of the T gene were produced. Various parts of the N-terminus of the T protein were fused with the EnhancedGreenFluorescentProtein (EGFP). The EGFP was here fused to the C-terminus of the T protein. The fusion protein which comprised the unchanged N-terminus of the T protein (putative signal sequence with lipophilic membrane domain) was actually incorporated into the nuclear membrane. However, the fusion construct from which the putative signal sequence and the lipophilic domains lack, was not incorporated into the nuclear membrane and accumulated in the cytoplasm. This showed that the N-terminus of the T protein is necessary and suffices to result in a localization within the nuclear membrane. In order to show that the T protein is actually localized in the nuclear membrane, antibodies were generated against a peptide sequence of the T protein. Immunohistochemical studies of tissues of man, mouse and rat were carried out with these antibodies. It showed that the antibody detects a protein which is localized in the nuclear membrane. Since it is difficult to differentiate by means of a light microscope whether the protein is localized in the nuclear membrane or

the nucleus itself, further analyses were made using the high-resolution method of electron microscopy. By this it was possible to clearly show that the T protein is localized in the nuclear membrane. As a detection reaction a second antibody was used here to which the enzyme horseradish peroxidase was coupled and which resulted in a color reaction (DAB). The stain or coloring formed can be seen in the electron-microscopic pictures only on the cytoplasmic side of the nuclear membrane. This indicates that the antibody recognizes an epitope of the T protein which is accessible from the cytoplasmic side for the antibody. The analysis of the immunohistochemical sections also showed that the antibody recognizes very specific neurons (cf. figure 24). The results of the analysis of the expression on a protein level by means of the antibody are highly consistent with the results of the analysis of the RNA expression. The mouse ortholog of the T gene was used in the RNA *in situ* analyses. Using the human T gene cDNA clones, murine cDNA clones of the mouse ortholog were initially isolated and sequenced for this purpose. The sequence analysis confirmed that the isolated cDNA clones was the mouse ortholog. Such a murine cDNA clone of the T gene was then used for the RNA *in situ* hybridization (cf. figures 25, 26, 27, 28). An expression analysis of the T gene of the mouse was then possible by means of this technique. The accurate analysis of the spatial-temporal expression profile showed that the T gene plays a decisive role in the generation, formation and maintenance of the nervous system in vertebrates. No expression can be detected during the early mouse embryogenesis on day 9.5 post conceptionem (pc = post conceptionem). On day 10.5 pc, it is possible to detect an expression in the ventral mesencephalon and in the telencephalon. In this stage there is also a strong expression in the connecting region of the mesencephalon and

telencepalon (forebrain-midbrain). An expression of the T gene in the telencephalon, in the ventral mesencephalon and in the myelencephalon can be detected on day 11.5 pc. An expression in neurons of the mantle zone of the developing brain and in the nuclei of the peripheral nerves is visible on day 12.5 pc. Furthermore, there is an expression in the myelencephalon, spinal cord and spinal ganglia. A minor expression is detectable in the mesencephalon and telencephalon. No expression is detectable e.g. in proliferating neurons in the subventricular layer or in the migrating neurons of the 'intermediate' zone. On day 14.5 pc, an expression in mesenchymal tissues, e.g. around the vertebra or in the region of developing bones, is also visible. A strong expression in all parts of the brain and the peripheral nervous system (e.g. spinal ganglia and nerve fibers of the tail) can be detected on day 16.5 pc. An expression in differentiating neurons of the mantle zone of the telecephalons can also be detected. Furthermore, an expression in neurons of the spinal cord and the spinal ganglia can be detected. When the brain develops after the birth, an expression in the olfactory bulb, in the cerebral cortex and in the developing hippocampus can be detected above all. A minor expression is found however in the coliculus and the developing cerebellum. A similar expression pattern exists in the fully developed brain.

Northern blots (cf. figure 23) were carried out to find out where the T gene or T2 or T3 gene are expressed. The T gene is expressed predominantly in the brain, hardly or not at all in the heart, lungs, placenta, liver, skeletal muscle, kidney or pancreas (irrespective of adult or fetal tissue). However, the T2 gene is virtually not expressed in the brain but strongly expressed in the heart (adult and fetal), adult liver, adult skeletal muscle and adult kidney. The T3 gene

is expressed in all tested tissues (adult and fetal heart, brain, liver, kidney: placenta, adult skeletal muscle, adult pancreas), except in fetal lungs.

Because of the discovery of the T gene and the detailed analysis of this gene with the information obtained therefrom a basis has been created for the development of fully novel medicaments and medicament compound classes. The bi-directional transport of molecules through the nuclear membrane is of decisive significance for the function of each eukaryotic cell. The information which is stored in the form of DNA (chromosomes) in the nucleus is transcribed into mRNA. However, the information is only translated into protein in the cytoplasm. If the transcribed information (mRNA) does not reach the cytoplasm, the information will be lost and dramatic disturbances may occur within the cell. This transport is, however, no one-way street. It is likewise important that certain substances and proteins reach the nucleus so as to maintain the function of the cell. If a transcription factor, for example, which - like the other proteins - is formed in the cytoplasm does not reach the cell nucleus, it cannot trigger the transcription of the other genes. Dramatic disturbances of the events in the cell, which may even comprise the dying of the cell or the organism, are often accompanied by this. This shows clearly that nuclear pore proteins perform an extremely important function within the cell. The analysis of the T gene has now shown that the T protein is also incorporated into the nuclear membrane. It is interesting that the T protein is almost twice as large as the POM121 protein, i.e. it has a much greater binding capacity than the POM121 protein. The T protein is therefore very well suited to isolate possible binding partners which attach to the T protein, in particular to the C-terminus of the T protein.

The tissue-specific expression of the T gene shows strikingly that nuclear core proteins (in particular nuclear pore membrane proteins) do not have to be expressed in all cells and at all times like 'housekeeping' genes. The predominant expression of the T gene in the nervous system shows that the T protein in the nervous system performs a very specific function. The predominant expression of the T gene in the nervous system can now be used for the development of new medicaments and new medicament compound classes. New substances can now be isolated by means of the T protein, which influence deliberately the bi-directional transport in nuclear pores of the nervous system. The localization of the T protein within the nuclear membrane is in this case of major advantage. Chemical compounds can be tested by means of automated tests. Many pharmaceutical companies have suitable screening methods in which more than 200,000 chemicals can be tested. For this purpose, e.g. reporter assays (e.g. GFP fusion proteins, colored substances, etc.) can be used which show the successful transport of a molecule into the nucleus or into the cytoplasm. By this, new active substances can then be isolated which deliberately influence the transport of molecules into nuclear pores, in particular those of the nervous system.

Identifying and analyzing interactions between the T proteins according to the invention (T, T2, T3 protein) or peptides or fragments thereof and possible binding partners which may represent active substances within the above-mentioned meaning, can happen e.g. with the "yeast-two-hybrid system" (Fields, Nature 340, pages 245-247, (1989)). This system is based on the discovery that cellular transcription activators, such as GAL4 or lexA from yeast,

can be separated into two independent functional domains. Both domains are usually part of a protein in the cell nucleus of the yeast cell, which binds to certain activating sequences of different target genes and regulates the transcription thereof. In this connection, one domain, the DNA binding domain (BD), binds specifically to a certain DNA target sequence (upstream activating sequence) in the vicinity of the target promoter. The other domain, the activation domain (AD), increases the transcription rate of the target gene by interaction with the transcription initiation complex which is bound to the promoter of the target gene. In the "yeast-two-hybrid system", this structure is used by the transcription factors in modified form. The DNA binding domain (BD) of GAL4 or lexA is expressed there as fusion protein with a "bait protein or peptide" (here: T, T2 or T3 protein/peptide) in yeast cells. This fusion also has a nuclear localization signal by which it is transported into the cell nucleus of the yeast. The bait fusion protein binds therein to a target sequence (UAS) which is located in the employed yeast strain in the vicinity of the promoters of two reporter genes (e.g. auxotrophic marker (HIS3) and enzymatic marker (lacZ)). By this a constellation results in which the bait protein or peptide is exposed in direct spatial vicinity of the reporter gene promoter. Then, a second fusion protein is additionally expressed in the same yeast cell. It consists of the activation domain (AD) of GAL4 or lexA and a prey protein or peptide. It also has a nuclear localization signal. The prey fusion protein is thus also transported into the cell nucleus of the yeast. If the prey protein and the bait protein exposed on the UAS physically interact with each other, it becomes more likely statistically that the activation domain is located in the vicinity of the reporter gene promoter. This results in an increase of the

transcription of the reporter genes whose extent is proportional to the strength of interaction between bait and prey protein. In this case, e.g. a cDNA library and also a combinatorial peptide library are in consideration as the prey proteins.

The present invention also relates to a process of identifying inhibitors or enhancers of the T protein family according to the invention. For this purpose, the nucleic acid sequences or parts of these sequences, which are part of the T gene or the paralogs or orthologs thereof, are inserted in suitable vectors and used for transfecting or transforming cells, tissues or organisms. These changed cells, tissues or organisms are then used for identifying inhibitors or enhancers of the T protein or its paralog or ortholog proteins (e.g. T2 and T3) or proteins which interact directly or indirectly with these proteins. The inhibitors or enhancers identified by this approach can be used for pharmaceutical active substances or medicaments or for the production thereof and for the treatment of diseases such as cancer, neurological and psychiatric diseases and injuries of the nervous system. In the case of injuries of the nervous system, innate damage of the nervous system or the degenerative diseases of the nervous system, it is possible to support deliberately by this treatment *inter alia* the neuronal regeneration or improve the interconnection of individual nervous regions (used for *inter alia* Alzheimer's disease, Parkinson's disease, schizophrenia, manic-depressive diseases, autism, mental retardation). The present invention provides the possibility of testing the substances or therapeutic agents suitable to enhance or reduce the effect of the T protein or the family of the T proteins. In particular, the changed nuclear pore properties which are influenced by the proteins T and T3 can

be detected by suitable screening methods. The latter include e.g. visualization of the bi-directional transport through the nuclear pore or the detection of a modified transcription of cellular or reporter genes. Substances or therapeutic agents can also be identified which inhibit or promote the effect of proteins which are directly or indirectly involved in the effect of the T protein or the family of the T proteins. Substances or therapeutic agents which show an enhancement or reduction of the effect of the T protein (or T2 or T3) in the above-mentioned screening methods, can be used to determine whether the enhancement or the reduction of the effect of the T protein results in therapeutically desired effects. Above all the inhibition of the growth or spreading of tumor cells or the support of neuronal regeneration, e.g. after injuries of the nerves (*inter alia* paraplegia and head-brain trauma), are counted thereamong. The identified substances can then be used as medicaments or for the production of these medicaments. Due to these medicaments it is then possible to inhibit or block spreading of the disease-inducing cells and thus control or clear up the disease on the whole. An important application of these medicaments is *inter alia* preventing the growth and spreading of tumor cells. In addition thereto, the identified active substances are used as medicaments which stimulate deliberately the growth of certain cells. By this it is then possible to regenerate cells or structures of the nervous system damaged by injury or degenerative processes. The T protein (or T2 or T3) can also be used in screening methods allowing not only to detect the changed nuclear pore properties but also to identify prior or subsequent or parallel signal cascades. By this it is possible to identify e.g. tyrosine kinases or tyrosine phosphatases which regulate proteins which in turn influence directly or indirectly the action of the T protein (or T2 or T3). As a

result, suitable targets for the positive influence of the events in the cells can be recognized and characterized. Furthermore, the T protein, although it occurs as a nuclear pore protein, is significant for the interactions with filaments of the cell, e.g. microtubuli and actin. These interactions can now be studied, e.g. by fusion proteins of the T protein with the EGFP protein. Cells which were stably or transiently transformed or transfected with constructs for such fusion-reporter proteins, can be incubated with substances or pharmaceutical preparations to identify substances which enhance or reduce the interaction of the T protein with filaments such as the actin filaments or the microtubuli. As a result, it is possible to isolate active substances which positively influence *inter alia* the growth of nerve cells or the inhibition of the growth of tumor cells. For example, immunoprecipitation has to be mentioned as a method of identifying such possible active substances. Proteins can be isolated by this which bind to the T protein family. Further immunoprecipitations can then be carried out with these proteins to isolate new proteins which then no longer interact directly with the T protein.

The present invention also relates to a method of identifying further proteins which play a role in the development and function of the nervous system and/or are a nuclear pore protein, the method comprising the steps of:

- (a) producing an antibody against a protein of the T family (T, T2 or T3 protein),
- (b) contacting a cell extract with the antibody and identifying the antibody/protein complex,

- (c) analyzing the complex to identify a protein which has bound to the protein of the complex and is no antibody, and
- (d) optionally repeating steps (a) to (c) to identify further proteins of this function.

The invention is described in more detail by means of the figures, which show:

Figure 1: human cDNA sequence (gene T) and derived amino acid sequence

Figure 2: human genomic DNA sequence (gene T)

Figure 3: human genomic DNA sequence (gene T)

Figure 4: human genomic DNA sequence (gene T)

Figure 5: human genomic DNA sequence (gene T)

Figure 6: human genomic DNA sequence (gene T)

Figure 7: human genomic DNA sequence (gene T)

Figure 8: human genomic DNA sequence (gene T)

Figure 9: partial murine cDNA sequence (gene T) and derived amino acid sequence

Figure 10: partial murine genomic DNA sequence (gene T)

Figure 11: partial human cDNA sequence (gene T2) and derived amino acid sequence

Figure 12: partial murine cDNA sequence (gene T2) and derived amino acid sequence

Figure 13: partial murine cDNA sequence (gene T2) and derived amino acid sequence

Figure 14: splicing variant of the human T gene with derived amino acid sequence

Figure 15: splicing variant of the human T gene with derived amino acid sequence

Figure 16: partial human cDNA sequence (gene T2) with derived amino acid sequence

Figure 17: partial human cDNA sequence (gene T3; protein isoform 1) with derived amino acid sequence

Figure 18: partial human cDNA sequence (gene T3; protein isoform 2) with derived amino acid sequence

Figure 19: partial murine cDNA sequence with derived amino acid sequence (gene T3)

Figure 20: oligonucleotide and peptides (T gene)

Figure 21: sequence comparison within the T family

Figure 22: protein alignment of POM121 protein and T protein

Figure 23: Northern blot analysis

Figure 24: immunohistochemical studies and electron-microscopic pictures

Figure 25: *in situ* hybridization with embryonal RNA

Figure 26: *in situ* hybridization with RNA from brain

Figure 27: *in situ* hybridization with RNA from fetal brain

Figure 28: *in situ* hybridization with RNA from nerve tissues of mouse

Figure 29: comparison of the coiled-coil regions between CLIP protein, T protein and POM121

Figure 30: hydrophobicity blot for POM121, T protein and T3 protein.

The following clones were deposited with the DSMZ (*Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH*) [German-type collection of microorganisms and cell cultures], Mascheroder Weg 1b, Braunschweig, according to the Budapest treaty on August 18, 1998:

- clone JFC277 (DSM12371); human cDNA; represents the human cDNA sequence of Bp 1218-3690
- clone JFC405 (DSM12372); human cDNA; represents the human cDNA sequence of Bp 1-1891
- clone JFC601 (DSM12373); murine cDNA; represents the murine cDNA sequence of Bp 225-3026
- clone JFC950 (DSM12374); human genomic clone; represents human genomic sequence

- clone JFC955 (DSM12375); human genomic clone; represents human genomic sequence; comprises start of the cDNA sequence
- clone JFC N2112 (DSM12376); human genomic clone; was fully sequenced. The sequence is shown in figure 2 and contains the sequence of Bp 1756-4228 of the human cDNA sequence.

The following clone was deposited with DSMZ according to the Budapest treaty on February 2, 1999:

- clone JFC-BN27 (DSM 12659); contains the sequence of Bp 4370-8690 of the human cDNA sequence.

The following clone was deposited with the DSMZ according to the Budapest treaty on February 19, 1999:

- clone JFC-BN20 (DSM 12698); contains the sequence of Bp 2025-6280 of the human cDNA sequence

The following clone was deposited with the DSMZ according to the Budapest treaty on February 1, 2000.

- cDNA clone pL70 (DSM13270); represents essential parts of the gene T3.

The sequences shown in figures 2 to 8 originates from clones JFC955 (DSM 12375) and JFC950 (DSM 12374). The sequence shown in figure 1 originates from clones JFC277 (DSM 12371), JFC405 (DSM 12372) and JFC-BN27 (DSM 12659) and JFC-BN20 (DSM 12698). The sequence shown in figure 9 originates from the clone JFC610 (DSM12373).

The invention is further described by means of the following embodiment.

### **EXAMPLES**

As to the methods employed reference is also made to Sambrook, J., Fritsch, E.F. and Maniatis, T. (Molecular Cloning; A Laboratory Manual; second edition; Cold Spring Harbor Laboratory Press, 1989) and Current Protocols in Molecular Biology (John Wiley and Sons, 1994-1998), the below techniques, in particular preparation of DNA or RNA or Northern blot, being sufficiently known to, and mastered by, the person skilled in the art.

Before it is described in detail how the experiments are carried out, the operating strategy is to be explained first.

When screening for genes triggering diseases of the CNS (e.g. neurodegenerative diseases, mental retardations, tumoral diseases of the CNS) in the mutated state, 23 cDNA clones were isolated from a human fetal brain cDNA library (Stratagene company, Heidelberg). A human fetal brain cDNA library was used as a starting material, since it was assumed that genes which play a role in the development of the CNS and in particular of the brain are present in a fetal brain cDNA library. However, since what is called housekeeping genes (genes expressed in most tissues) are also expressed in the CNS, it was tested whether the select cDNA clones originate from genes having a CNS-specific expression. For this purpose, the cDNA pieces ('inserts') contained in the individual cDNA clones were isolated and used for hybridization with Northern blots. The employed

Northern blots comprised polyA RNA from different human tissues (e.g. brain, skeletal muscle, liver and kidney) and various development stages (fetal and adult tissues). Since as mentioned above not only brain-specific genes are expressed in the brain, the hybridization with the Northern blots was used to identify cDNA clones which are expressed above all in the brain and not so much in other tissues. Due to this differential analysis it was possible to identify a cDNA clone which has a brain-specific expression pattern. Using this cDNA clone, the entire mRNA sequence for the new protein encoded therein could be isolated and deciphered (gene T with protein TP encoded therein) by repeated hybridization of the fetal cDNA library.

**EXAMPLE 1: Identification of the T genes**

**1. Titration of the cDNA libraries**

In order to ensure an effective infection, it was initially necessary to produce phage-competent bacteria in an overnight culture. The magnesium ions contained in the medium induce the maltose receptor of the bacteria to which the phage binds to infect the bacterium.

Performance:

Charge 50  $\mu$ l *E. coli* XL1-Blue in 50 ml LB broth, the medium being admixed with  $MgSO_4$  in a concentration of 10 mM. Incubate overnight at 30°C and 220 rpm. Centrifuge off the bacteria at 4°C and 1000 xg for 10 min. Resuspend in 25 ml 10 mM  $MgSO_4$ . The thus produced phage-component bacteria could be stored at 4°C for up to one week.

**2. Culturing the cDNA libraries**

For culturing the library, Baltimore Biological Lab. (BBL) agar plates and BBL top agarose had to be prepared. The phages (human or murine cDNA library, Stratagene company) were mixed with SM medium to a dilution of 1:10<sup>3</sup> and 1:10<sup>4</sup> to obtain individual plaques after the culturing.

Performance:

For the BBL agar (pH 7.2) 10 g BBL trypticase, 5 g NaCl and 10 g Select agar were weighed and filled to 1 l with H<sub>2</sub>O. The agar is dissolved by autoclaving. After cooling to about 60° pour the plates. The plates are preheated to 37°C prior to their use to avoid premature solidification of the top agarose. The BBL top agarose (pH 7.2) was prepared with 10 g BBL trypticase, 5 g NaCl, 6.5 g agarose and 10 ml 1 M MgSO<sub>4</sub> solution. Dissolve by autoclaving and provide in the water bath to 41°C. Add 15 µl of the above indicated dilute phage solution and 250 µl of the competent XL-1 bacteria in a 15 ml Falcon tube. Incubate at room temperature for 20 minutes. Add 10 ml BBL top agarose, swivel and place on the heated agar plate. The top agarose layer is solid after about 20 minutes and the plates can be stacked with the agar side up. Incubation is carried out overnight at 37°C. The plates can be stored at 4°C after expired incubation time or can be used directly for transferring the phage plaques. Carefully close the plates for storing them together with a chloroform-soaked cloth in plastic bags. The chloroform prevents the growth of cryophilic bacteria and fungi.

**3. In vivo excision**

The employed cDNA libraries (human and murine fetal brain cDNA library; Stratagene company, Heidelberg) were cloned in the vector λ-ZAPII. Due to this there was the possibility of circumventing the subcloning of the phage insert in a plasmid vector. This protocol permits to transfer cDNA which

is located as insert in the  $\lambda$ -ZAPII vector into an insert in simple way by an *in vivo* preparation which is now found in the plasmid Bluescript SK(-). In principle, this preparation serves for introducing by a helper phage information for proteins which permit DNA amplification only in the region of the phage genome, which have the genetic information for the plasmid with cDNA insert. For the most part, the method was carried out in accordance with the protocol of the manufacturer (Stratagene).

In particular, culturing was made such that individual phage plaques were on the plate. Then, the *in vivo* excision protocol was carried out with these individual plaques. The plasmid DNA and its plasmid inserts were isolated from the bacterial clones and subsequently hybridized with Northern blots. The selection of further clones to be studied was based on the expression pattern in the Northern blots.

### Performance:

Mix 100  $\mu$ l of a single phage  $\lambda$ -ZAPII clone with 200  $\mu$ l XL1 bacteria and 2  $\mu$ l helper phages (contained in the Stratagene kit). Shake for 15 min. at 37°C and 80 rpm, the specific attachment of both phage types to the host bacterium taking place. Add 3 ml LB broth. Incubate for 2 h at 37°C and 200 rpm. The DNA replication of the plasmid contained in the  $\lambda$ -ZAPII, its circularization and the packing into coat proteins take place and discharge from the bacterium occur during this time. Heat to 70°C for 20 minutes. Thereafter, centrifuge at 4000 g for 15 minutes. This kills the still remaining bacteria and separates their fragments from the plasmids existing in the phage coat, which are found in the supernatant. Add 1  $\mu$ l thereof to 200  $\mu$ l SOLR host cells, incubate at 37°C for 15 minutes. Plate 100  $\mu$ l onto LB/Amp plates. Store at 37°C overnight. The then grown bacterial

clones contain the plasmid with the corresponding cDNA insert. A mini-prep DNA preparation was carried out each.

#### **4. "random primed" DNA labeling**

The radioactive labeling of the double-stranded insert DNA of the cDNA clone was carried out as follows for the further isolation of overlapping cDNA clones:

##### Performance:

Dissolve 100 ng DNA in a volume of 12  $\mu$ l H<sub>2</sub>O for a typical labeling batch. 10-minute heating to 95°C effects the denaturation of the DNA into single strands. Store the preparation on ice to prevent reassociation of the two complementary DNA strands. Complete the reaction batch by 4  $\mu$ l OLG (oligo-labelling buffer), 1  $\mu$ l Klenow (1U) and 2.5  $\mu$ l  $\alpha$ -<sup>32</sup>P-dCTP and 2.5  $\mu$ l  $\alpha$ -<sup>32</sup>P-dATP. Incubate at room temperature overnight. Based on the hexanucleotides attached to a single strand, the formation of the complementary strand takes place during this time by the Klenow fragment of the *E. coli* DNA polymerase I. The DNA is labeled radioactively by incorporating  $\alpha$ -<sup>32</sup>P-dCTP and the  $\alpha$ -<sup>32</sup>P-dATP.

#### **5. Separation of non-incorporated radioactive nucleotides**

The non-incorporated nucleotides were separated by means of a personally prepared sephadex G-50 column. The separation principle of the column is based on the exclusion chromatography. The smaller non-incorporated nucleotides fit into small pores of the column material while the DNA is locked out. The volume in which the nucleotides may move is thus greater than the volume available to the DNA. If a mixture of DNA and nucleotides is placed on the column, the DNA runs through the column faster than the nucleotides. This permits the separation of non-incorporated nucleotides.

**Performance:**

A Pasteur pipette was closed with a small glass bead. Fill the Pasteur pipette with sephadex G-50 ("fine") dissolved in water until the filling material is 5 cm below the top edge of the Pasteur pipette. Rinse the column 2 times with TE. Apply the above radioactive labeling batch. Add 320  $\mu$ l TE. Discard the solution which has run through the column. Place an Eppendorf tube below the column. Add 350  $\mu$ l TE. Collect the radioactive solution run through the column.

**6. Plaque "blot"**

The plaque "blot" was made to analyze the cDNA library to make accessible the cDNA in the phage clones to hybridization.

**Performance:**

Place a labeled hybond-N membrane provided with an inscription in air bubble-free manner on the plate with the phage plaques for one minute. The labeling pattern was transferred. Place it on a Whatman paper soaked with denaturing solution (0.5 M NaOH; 1.5 M NaCl) for 10 minutes. Neutralize in 50 mM phosphate buffer for 10 minutes. The rests of the bacterial layer are wiped off with slight pressure using a phosphate buffer-soaked Kleenex cloth. The filters are spread at room temperature for drying. Thereafter, the filters were baked at 90°C for 1 h.

**7. Hybridization**

The hybridization is based on the binding of complementary, single-stranded nucleic acids. For this purpose, the DNA to be studied was immobilized on a membrane and hybridized with a radioactively labeled probe. The complementary binding is maintained even after washing off the non-specifically adhering probes and can be made visible by means of

autoradiography. Single-stranded molecules were incubated during the hybridization under salt and temperature conditions which support the formation of base-paired double strands. A decisive factor in the association and dissociation kinetics are the hydrogen bridge bonds between the base pairs G-C and A-T. The hybridization reaction is influenced by changing the temperature and the salt and sample concentrations.

Performance:

First, prehybridize the DNA filters in hybridization solution (0.5 M NaPi (pH 7.2); 7 % SDS; 0.2 % BSA; 0.2 % PETG 6000; 0.05 % polyvinyl pyrrolidone 360000; 0.05 % Ficoll 70000; 0.5 % dextrane sulfate) with a 0.1 ml/cm<sup>2</sup> at 65°C. For this purpose, incubate the filters in a plastics box in a shaking water bath at 65°C for a period of at least 1 h. Discard the prehybridization solution. Place the radioactively labeled sample (see above items 4. and 5.) with 0.5 ml/cm<sup>2</sup> of hybridization solution (65°C) on the filters. The activity of the sample should not drop below 50 cpm, measured at a distance of 40 cm. The hybridization takes place overnight at 65°C (human cDNA library) or 55°C (interspecies hybridizations man-mouse and for isolating the homologous genes). Wash the filters two times for 30 minutes with about 500 ml wash buffer in a shaking bath at 65°C (55°C). Autoradiography was then carried out.

**8. Autoradiography**

The filters were packed in plastic foodwrap. The autoradiography was made at -80°C in an X-ray cassette containing a reinforcing film made of calcium tungstate. The exposure is 30 minutes to several days, depending on the strength of the signal.

The complete mRNA which codes for the protein of the T gene could be isolated by means of the above mentioned techniques. Furthermore, using cDNA clones of this newly isolated T gene it was possible to isolate two further genes (T2 and T3) which have distinct homologies with this gene. For this purpose, the above mentioned techniques were used again. For isolating the related genes T2 and T3, the hybridization temperature was lowered to 55°C.

#### **EXAMPLE 2: Northern blot**

The 'multiple tissue Northern blots' were purchased from the CLONTECH company (Palo Alto, California, U.S.A.) and used in accordance with the instructions from the manufacturer. The respective DNA samples of the genes T, T2 and T3 were labeled radioactively and hybridized with the Northern blots. The sequence of bp 1-4200 of figure 1 was used for the analysis of the expression pattern on a Northern blot level. For the gene T3 the sequence of bp 1310-4870 of figure 17 was used for hybridization. The sequence of bp 3120-4230 of figure 16 was used for the gene T2. The "random priming" method was used for the radioactive labeling of double-stranded DNA.

##### a) Random priming:

Dissolve 100 ng DNA in a volume of 12  $\mu$ l for a typical labeling batch. 10-minute heating to 95°C effects the denaturation of the DNA into single strands. Store the batch on ice to prevent reassociation of the two complementary DNA strands. Complete the reaction batch by 4  $\mu$ l OLB, 1  $\mu$ l Klenow (1U) and 2.5  $\mu$ l  $\alpha$ -<sup>32</sup>P-dCTP and 2.5  $\mu$ l  $\alpha$ -<sup>32</sup>P-dATP. Incubate at room temperature overnight. Based on the hexanucleotides attached to a single strand, the formation of the complementary strands takes place during this time by

the Klenow fragment of the *E. coli* DN polymerase I. The DNA is labeled radioactively by the incorporation of the  $\alpha$ -<sup>32</sup>P-dCTP and the  $\alpha$ -<sup>32</sup>P-dATP.

The non-incorporated nucleotides were separated by means of a personally prepared sephadex G-50 column. The separation principle of the column is based on the exclusion chromatography. The smaller non-incorporated nucleotides fit into small pores of the column material while the DNA is locked out. The volume in which the nucleotide may move is thus greater than the volume available to the DNA. If a mixture of DNA and nucleotides is placed on the column, the DNA runs through the column faster than the nucleotides. This permits the separation of non-incorporated nucleotides. For this purpose, a Pasteur pipette is closed with a small glass bead. Fill the Pasteur pipette with sephadex G-50 ("fine") dissolved in water until the filling material is 5 cm below the top edge of the Pasteur pipette. Rinse the column 2 times with TE. Apply the above radioactive labeling batch. Add 320  $\mu$ l TE. Discard the solution which has run through the column. Place Eppendorf tube below the column. Add 350  $\mu$ l TE. Collect the radioactive solution run through the column.

b) Hybridization:

The Northern blots were hybridized as described below. First, the Northern blots were prehybridized at 65°C in 10 ml hybridization solution (350 ml 20 % SDS, 500 ml 1 M phosphate buffer, pH 7.2; 150 ml distilled water). For this purpose, the Northern blots were incubated in a glass tube in a hybridization roll-over-type furnace at 65°C for a period of 6 h.

The prehybridization solution was discarded. The radioactively labeled sample was placed with 10 ml hybridization solution (65°C) on the filters.

The hybridization was carried out at 65°C overnight. The filters were then washed two times for 30 min. with about 500 ml wash buffer (80 ml 1 M phosphate buffer, pH 7.2; 100 ml 20 % SDS, 1820 ml distilled water) at 65°C in a shaking bath.

c) Autoradiography

The filters were welded into plastic film. The autoradiography was made at -80°C in an X-ray cassette which contained a reinforcing film of calcium tungstate. Exposure was 1 to 4 days depending on the strength of the signal.

The results of the Northern blots carried out are shown in figure 23.

**EXAMPLE 3: RNA *in situ* hybridization**

Embryos in various development stages were isolated from pregnant NMRI mice. The embryos and other tissue samples were fixed overnight with 4 % paraformaldehyde in PBS at 4°C. 10 µm freezing sections of the embryos were transferred to slides coated with 3-aminopropyl triethoxysilane. Sense strand ("sense") and antisense strand ("antisense") samples were produced by transcription with a-<sup>35</sup>S-UTP with a specific activity of >10<sup>9</sup> decays per minute/µg. For this purpose, the linearized mouse T gene cDNA clone from figure 9 was transcribed with T7 or Sp6-RNA polymerase. The sample length was reduced by alkaline lysis to 150 to 200 nucleotides. The slides were prehybridized at 54°C in a solution containing 50 % formamide, 10 % dextrane sulfate, 0.3 M NaCl, 10 mM Tris, 10 mM sodium phosphate, pH 6.8, 20

mM dithiothreitol, 0.2 % Denhardt's solution, 0.1 Triton X-100, 0.1 mg/ml Escherichia coli RNA and 0.1 mM non-radioactive a-S-UTP. The  $^{35}$ S-labeled sample ( $8 \times 10^4$  decays per minute per ml) were added to the hybridizing mixture for the hybridization and the hybridization was then continued for 16 h at 54°C in a humid chamber. The slides were then washed in the hybridization solution for 2 hours. The remaining non-hybridized RNA sample was then digested using RNase A. Thereafter, the slides were washed for 30 minutes at 37°C with 2x SSC, 0.1 % SDS and for 30 minutes with 0.1x SSC, 0.1 % SDS. Then, the slides were dehydrated with increasing ethanol concentrations. The slides were covered with Ilford K5 autoradiography emulsion. After 1 to 2 weeks of exposure at 4°C, the slides were incubated in Kodak D19b developer and dyed with Giemsa. The sections were analyzed in dark field and bright field illumination with a Zeiss SV8 stereomicroscope and an Axiophot microscope and photographed with an Agfa ortho black-and-white film.

The results of the RNA *in situ* hybridization are shown in figures 25, 26, 27 and 28.

Figure 25: expression of the murine T gene during the mouse embryogenesis. Bright field (a,c,e,g) and dark field pictures (b, d, f, h) of horizontal (a,b) and sagittal sections (c-h) through a 10.5 (a,b), 12.5 (c,d), 14.5 (e,f) and 16.5 (g,h) dpc embryo (dpc = days post conceptionem) which were hybridized with an antisense ribo sample of the murine T gene. Dec = decidua, g = guts, he = heart, lab = labyrinth, li = liver, me = myelcephalon, sc = spinal cord, sga = spinal ganglia, sb = tooth bud, te = telencephalon. Bar = 1 mm.

Figure 26: Expression of the murine T gene in the postnatal brain. Bright field (a,d) and dark field pictures (b,c,e,f) of horizontal sections through an 1 wpn (weeks post natalis) and 6 wpn head, which were hybridized with a T gene antisense (b,e) and a sense sample (c,f). cd = cerebellum, cor = cortex, cos = colliculus, ey = eye, hi = hippocampus, ne = nasal epithelium, ob = olfactory bulb, bar = 1 mm.

Figure 27: Greater enlargement of the 10.5 dcp embryo of figure 25 a,b. The arrows point to a region of little expression in the somites (arrows in b). An intense expression can be seen in the region between mesencephalon and telencephalon ("forebrain-midbrain junction"). Aod = aorta dorsalis, me = mesencephalon, sc = spinal cord, te = telencephalon. Bar = 100  $\mu$ m.

Figure 28: Expression of the T gene during the development of the nervous system. Expression of the T gene in neurons of the mantle zone of the developing brain and in nuclei of peripheral nerves (arrow in b). No expression is visible in proliferating neurons in the subventricular layer or in migrating neurons of the intermediate zone (c,d). On day 16.5, an intense expression is visible in differentiating neurons of the mantle zone of the telencephalon (e,d). A minor expression is also visible in neurons of the spinal cord and the spinal ganglia (g,h). Furthermore, a minor expression is visible in an individual layer below the skin (g,h). iz = intermediate zone, mz = mantle zone, sc = spinal cord, sga = spinal ganglia, sk = skin, svl = subventricular layer, vn = ventricle. Bar = 100  $\mu$ m.

#### **EXAMPLE 4: Production of antibodies**

Using a synthetically produced peptide of the sequence "EKGEDPETRRMRTVKNIAD" animals are immunized to produce antibodies against the T protein as follows:

Immunization protocol for polyclonal antibodies in rabbits

600 µg purified KLH-linked peptide in 0.7 ml PBS and 0.7 complete or incomplete Freund's adjuvant are used per immunization:

Day 0: 1<sup>st</sup> immunization (complete Freund's adjuvant)  
Day 14: 2<sup>nd</sup> immunization (incomplete Freund's adjuvant; icFA)  
Day 28: 3<sup>rd</sup> immunization (icFA)  
Day 56: 4<sup>th</sup> immunization (icFA)  
Day 80: bleeding to death.

The rabbit serum is tested in an immunoblot. For this purpose, the protein used for the immunization is subjected to SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter (cf. Khyse-Andersen, J., J. Biochem. Biophys. Meth. 10 (1984), 203-209). The Western blot analysis was carried out as described in Bock, C.-T. et al., Virus Genes 8, (1994), 215-229. For this purpose, the nitrocellulose filter is incubated with a first antibody at 37°C for one hour. This antibody is the rabbit serum (1:10000 in PBS). After several wash steps using PBS, the nitrocellulose filter is incubated with a second antibody. This antibody is an alkaline phosphatase-coupled monoclonal goat anti-rabbit IgG antibody (Dianova company) (1:5000) in PBS. 30 minutes of incubation at 37°C are followed by several wash steps using PBS and subsequently by the alkaline phosphatase detection reaction with developer solution (36 µM 5'-bromo-4-chloro-3-indolylphosphate, 400 µM nitro blue tetrazolium, 100 mM Tris-HCl, pH 9.5, 100 mM

NaCl, 5 mM MgCl<sub>2</sub>) at room temperature until bands become visible.

It shows that polyclonal antibodies according to the invention can be prepared.

Immunization protocol for polyclonal antibodies in chickens

100 µg of purified KLH-linked peptide in 0.8 ml PBS and 0.8 ml of complete or incomplete Freund's adjuvant are used per immunization.

Day 0: 1<sup>st</sup> immunization (complete Freund's adjuvant)  
Day 28: 2<sup>nd</sup> immunization (incomplete Freund's adjuvant;  
icFA)  
Day 50: 3<sup>rd</sup> immunization (icFA)

Antibodies are extracted from egg yolk and tested in a Western blot. Polyclonal antibodies according to the invention are detected.

Immunization protocol for monoclonal antibodies in mice

250 µg of purified KLH-coupled peptide in 0.25 ml PBS and 0.25 ml of complete or incomplete Freund's adjuvant are used per immunization. The peptide is dissolved in 0.5 ml (without adjuvant) in the 4<sup>th</sup> immunization.

Day 0: 1<sup>st</sup> immunization (complete Freund's adjuvant)  
Day 28: 2<sup>nd</sup> immunization (incomplete Freund's adjuvant;  
icFA)  
Day 56: 3<sup>rd</sup> immunization (icFA)  
Day 84: 4<sup>th</sup> immunization (PBS)  
Day 87: fusion.

Supernatants of hybridomas are tested in a Western blot. Monoclonal antibodies according to the invention are identified.

**EXAMPLE 5: immunohistochemical studies**

The immunohistochemical studies shown in figure 24 were made with an affinity-purified polyclonal rabbit antibody, produced above, against the T protein (referred to as first antibody below). Mouse brain was removed and treated as follows:

1<sup>st</sup> day

section thickness 6-10 µm, common fixation on slides, storage at -80°C for up to about 2 months

Take out the sections the evening before and allow them to dry at room temperature overnight

Rinse slides in PBS, pour off, rinse once again, thereafter allow to stand in PBS for 10 min.

Take out slides and wipe off the liquid around the tissue using a cloth.

Encircle using PAP-PEN (protein-glycerol; Dako company) so that no more liquid can flow out.

Add 100 µl peroxidase blocking solution (Dako company, Hamburg), incubate for 20 minutes.

Rinse slides in PBS, pour off, rinse again, thereafter allow to stand in PBS for 10 min.

Take out slides and wipe off the liquid around the tissue using a cloth.

Prepare an 1:10 dilution of normal (sheep) serum in PBS (e.g. sheep Dako X0503, Dako company, Hamburg), add 100 µl thereof and incubate for 20 minutes.

Rinse slides in PBS, pour off, rinse again, thereafter allow to stand in PBS for 10 minutes.

Take out slide and wipe off the liquid around the tissue using a cloth.

Add first antibody in a dilution of 1:100.

Add 100  $\mu$ l of the first antibody (in PBS) and incubate in a refrigerator in a humid chamber overnight. Control: without first antibody.

2<sup>nd</sup> day

Take humid chamber out of the refrigerator and allow to stand at room temperature. Rinse slide in PBS, pour off, rinse again, thereafter allow to stand in PBS for 10 minutes, when many slides are analyzed wash two times with PBS.

Take out slides and wipe off the liquid around the tissue using a cloth.

Prepare a 1:100 dilution of second antibody "antirabbit biotinylated" (Amersham company, Braunschweig) in PBS and add 100  $\mu$ l thereof.

Incubate in a humid chamber at room temperature for 45 minutes.

Rinse slides in PBS, pour off, rinse again, thereafter allow to stand in PBS for 10 minutes.

Take out slide and wipe off the liquid around the tissue using a cloth.

Prepare a 1:100 dilution of streptavidine peroxidase (streptavidine horseradish) (Amersham company, Braunschweig) with PBS and add 100  $\mu$ l thereof.

Incubate in a humid chamber at room temperature for 45 minutes.

Rinse slides in PBS, pour off, rinse again, thereafter allow to stand in PBS for 10 minutes.

Take out slides and wipe off the liquid around the tissue using a cloth.

Staining: Add one drop chromogen per ml buffer just before the use. Vortex and place in the dark.

Add 100 µl staining solution (Dako company, Hamburg).

Finally, stain the control. Incubate for about 2 minutes.

Incubate slides in water. Inspect under a microscope.

Place 1-2 drops of crystal Mount on the section. If there is an air bubble, suck it off with a paper handkerchief.

The rest of the slide is wiped doff using HCl-EtOH to remove the stain.

Place a line of adhesive (Eukitt) on the cover glass. Press the cover glass onto the slide without producing air bubbles.

The enzyme in the second antibody results in a dye formation (DAB) so that the T protein can be detected.

Figure 24 (a-d): Light-microscopic pictures which show that the T protein is localized in or at the nucleus of the cell. The electron-microscopic picture in e shows that the T protein is not localized in the nucleus but in the membrane. The pictures are highly consistent with a function as a membrane-terminal nuclear pore protein. The arrows in e show the stain formed which can be seen on the cytoplasmic side of the nuclear membrane.

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